UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY

AND POLLUTION PREVENTION

MEMORANDUM

DATE: September 22, 2016

SUBJECT: **Nitrapyrin**: Fifth Report of the Cancer Assessment Review Committee

PC Code: 069203
Decision No.: N/A
Petition No.: N/A
Risk Assessment Type: Cancer Assessment
TXR No.: 0057491
MRID No.: N/A

DP Barcode: N/A
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Case No.: N/A

CAS No.: N/A
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FROM: Christopher Schlosser, Executive Secretary
Cancer Assessment Review Committee
Health Effects Division (7509P)

THROUGH: Karlyn Middleton Co-Chair
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TO: Nancy McCarroll, Toxicologist
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The Cancer Assessment Review Committee met on May 25, 2016 to re-evaluate the cancer classification of Nitrapyrin in accordance with the *EPA's Final Guidelines for Carcinogen Risk Assessment* (March, 2005). Attached please find the final Cancer Assessment Document.

CANCER ASSESSMENT DOCUMENT

EVALUATION OF THE CARCINOGENIC POTENTIAL/MODE OF ACTION

FOR MOUSE LIVER TUMORS

NITRAPYRIN

PC Code 06923

September 22, 2016

CANCER **A**SSESSMENT **R**EVIEW **C**OMMITTEE

HEALTH EFFECTS DIVISION

OFFICE OF PESTICIDE PROGRAMS

Contents

I. Executive Summary

The Cancer Assessment Review Committee met on May 25, 2016 to re-evaluate the cancer classification of Nitrapyrin in accordance with the *EPA's Final Guidelines for Carcinogen Risk Assessment* (March, 2005). This is the fifth time that Nitrapyrin has been presented to either the Health Effect Division's Cancer Peer Review Committee (CPCR) or the Cancer Assessment Review Committee (CARC) to evaluate the carcinogenic potential of this nitrification inhibitor. Over the course of these meetings, multiple tumors (i.e., renal tumors in male rats, stomach, epididymis, or Harderian gland neoplasms in either male or female mice) were concluded to be either not treatment related or not relevant for the human risk assessment. At the fourth meeting, held December 2011, the CARC concluded, "The liver tumors in male and female mice, which were primarily benign, were treatment-related." The Committee also reviewed data to support a proposed mode of action for the liver tumors in mice. The CARC acknowledged that there was some evidence of mitogenesis through activation of the constitutive androstane receptor (CAR) nuclear receptor. However, the submitted data were not sufficient to support the proposed MOA for mouse liver tumors (Kidwell, 2012; TXR No. 0056262). Since that time, the Dow Company has submitted three new studies addressing this issue and an overview document detailing a human relevance framework analysis. On May 25, 2016, CARC reconvened to evaluate these submissions, which included the following:

1) A CAR Knockout assay in male wild type mice and CAR knockout mice (MRID 49677501);
2) An *in vitro* mouse and human primary hepatocyte cell proliferation assay (MRID 49677502);
3) An *in vitro* assay for evaluating suicide inhibition of CYP2B (MRID 49677503); and
4) An MOA and human relevance framework for mouse liver tumors (MRID 49677504).

These new studies were considered when reevaluating the data to support the proposed MOA for the mouse liver tumors. Using the CAR Knockout Assay in male mice as the critical study, CARC concluded that based on the dose and temporal concordance, the key events were established in the male mice at the tumorigenic dose of 250 mg/kg/day. Similarly, the associative events were established in male mice at 250 mg/kg/day and the female mice at $\geq$ 125 mg/kg/day (tumorigenic doses). However, the key events were not established in female mice since there are no mechanistic studies available. Therefore, **it was concluded that the available data adequately support a CAR mode of action in male mice only. The data are inadequate to support the key events to establish a definitive mode of action in female mice. Tumor induction in females occurred at a dose lower than that in males.**

In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, *2*005), CARC concludes that nitrapyrin should remain classified as "Suggestive Evidence of Carcinogenic Potential". The rationale for this decision is based on the following considerations:

- The liver tumor response induced by nitrapyrin occurred in male and female mice; no liver tumors were seen in the guideline 2-year carcinogenicity study in rats.
- The tumor response in both sexes occurred late in the course of treatment and was driven by adenomas.

- There is no concern for mutagenicity. The available mutagenicity studies for nitrapyrin were negative.

Data are sufficient to support the proposed MOA for liver tumors in male mice. However, a similar conclusion cannot be reached for the female mice with tumor induction apparent at a dose lower than the male mice. Accordingly, data are not sufficient to support the proposed MOA for female mouse liver tumors. The CARC determined that quantification of cancer risk using a non-linear approach (i.e., RfD) would adequately account for all chronic toxicity, including carcinogenicity, that could result from exposure to nitrapyrin.

II. BACKGROUND

The Cancer Assessment Review Committee met on May 25, 2016 to re-evaluate the cancer classification of Nitrapyrin in accordance with the *EPA's Final Guidelines for Carcinogen Risk Assessment* (March, 2005).This is the fifth time that Nitrapyrin has been presented to either the Health Effect Division's Cancer Peer Review Committee (CPCR) or the Cancer Assessment Review Committee (CARC) to evaluate the carcinogenic potential of this nitrification inhibitor. Over the course of these meetings, multiple tumors (i.e., renal tumors in male rats, stomach, epididymis, or Harderian gland neoplasms in either male or female mice) were concluded to be either not treatment related or not relevant for the human risk assessment. At the fourth meeting, held December 2011, CARC concluded that "The liver tumors, which were primarily benign, were treatment-related in the mouse." The Committee also acknowledged that there was some evidence of mitogenesis through activation of the constitutive androstane receptor (CAR). However, the submitted data were not sufficient to support the proposed MOA for liver tumors in mice (Kidwell, 2012; TXR No. 0056262). Since that time, the Dow Company has submitted three new studies addressing this issue and an overview document detailing a human relevance framework analysis. On May 25, 2016, CARC reconvened to evaluate these submissions, which included the following:

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4) An MOA and human relevance framework for mouse liver tumors (MRID 49677504).

These new studies have been reviewed and considered in the revaluation of the proposed MOA for mouse liver tumors.

III. EVALUATION OF LIVER TUMORS AND MECHANASTIC STUDIES

As stated above, CARC determined that the liver tumors induced by nitrapyrin were treatment-related in male and female mice, based on the following information:

A. Liver Tumors (extracted from TXR #0014131):

In the 2-year dietary cancer study, B6C3F1 mice (50/sex/dose) received nitrapyrin at dietary levels of 0, 125 or 250 mg/kg/day for 24 months (MRID 44231803). An additional 10 mice per sex per dose were designated for interim sacrifice at 12 months.

Data from the tumor analyses are presented in **Table 1**. As shown, male mice had significant differences in the pair-wise comparisons of the 250-mg/kg/day-dose group with the controls for hepatocellular adenomas (45/48, 94% vs. 12/49, 24% in the controls), and adenomas/carcinomas combined (46/49, 94% vs. 17/49, 35% in the controls, both at $p<0.01$). A significant ($p<0.01$) increasing trend for these tumors was also noted. Although the combined incidence (at 250 mg/kg/day) was driven by the incidence of adenomas, it had a malignant component (carcinomas: 12/49, 24% vs. 7/49, 14% in control). The incidence of adenomas at 250 mg/kg/day was outside the range for the historical control (14-54%).

Table 1. Nitrapyrin (N-Serve™) $B_6C_3F_1$ Mouse Study: Male Liver Tumors Rates[+] and Peto's Prevalence Test Results (p values)

Dose (mg/kg/day)			
	0	**125**	**250**
Adenomas (%) p =	12/49 (24) 0.000**	19/50 (38) 0.107	45[a]/48 (94) 0.000**
Carcinomas (%) p =	7/49 (14) 0.176	3/50 (6) NA	12[b]/49 (24) 0.188
Combined (%) p =	17[c]/49 (35) 0.000**	20[c]/50 (40) 0.244	46[d]/49 (94) 0.000**

[+] No. of tumor-bearing animals/No. of animals examined, excluding those that died or were sacrificed before observation of the first tumor.
[a] First adenoma, not in and interim sacrifice animal, observed at week 75, at 250 mg/kg/day.
[b] First carcinoma observed at week 72, at 250 mg/kg/day.
[c] Two of the animals in each of the 0 and 125 mg/kg/day groups has both an adenoma and a carcinoma.
[d] Eleven animals in the 250 mg/kg/day dose group had both an adenoma and a carcinoma.
Note: Two animals in the control group and 3 animals in the 250 mg/kg/day doses group, of the interim sacrifice groups had liver adenomas. No liver carcinomas were observed in any of the interim sacrifice males.
Significance of trend denoted at control. Significance of pair-wise comparison with control denoted at dose level. If *, then $p < 0.05$. If **, then $p < 0.01$.

In females, significant differences in the pair-wise comparisons of the 125- and 250-mg/kg/day groups with the controls for hepatocellular adenomas (27/48, 56% and 32/48, 67%, respectively vs. 6/47, 13% in controls), and adenomas and carcinomas combined (28/48, 58% and 33/48, 69%, respectively, vs. 6/47, 13% in controls), at $p < 0.01$ (**Table 2**). The combined rate was driven by the adenomas, and the incidence of adenomas at 125 and 250 mg/kg/day was outside the range for the historical control (4-26%). A significant ($p<0.01$) increasing trend for these tumors was also noted.

Table 2. Nitrapyrin (N-Serve™) $B_6C_3F_1$ Mouse Study: Female Liver Tumors Rates[+] and Peto's Prevalence Test Results (p values)

Dose (mg/kg/day)			
	0	**125**	**250**
Adenomas (%) p =	6/47 (13) 0.000**	27[a]/48 (56) 0.000**	32/48 (67) 0.000**
Carcinomas (%) p =	0/47 (14) 0.150	1[b] /48 (2) 0.505	2[b]/48 (4) 0.253
Combined (%) p =	6/47 (13) 0.000**	28/48 (58) 0.000**	33[c] /48 (69) 0.000**

[+] No. of tumor-bearing animals/No. of animals examined, excluding those that died or were sacrificed before week 54.

[a] First adenoma observed at week 75, at 125 mg/kg/day.

[b] First carcinoma observed simultaneously at week 105 in final sacrifice animals, doses 125 and 250 mg/kg/day.

[c] One animal in the 250 mg/kg/day dose group had both an adenoma and a carcinoma.

Note: There were no liver adenomas or carcinomas observed in any interim sacrifice animals. Significance of trend denoted at control. Significance of pair-wise comparison with control denoted at dose level. If *, then $p < 0.05$. If **, then $p < 0.01$.

B. Other Toxic Effects

The liver tumors in male and female mice were supported by increased liver weights and associated non-neoplastic lesions. Significant ($p<0.05$) and treatment-related increases in absolute and relative (to body weight) liver weight were observed in both sexes at both doses after 12 and 24 months. These increases ranged from 111 to 129 % of control at 125 mg/kg/day and 131 to 201% of control at 250 mg/kg/day. Findings from non-neoplastic liver lesions presented at the second CARC meeting included centrilobular or panlobular hepatocyte hypertrophy in all treated animals at 12 months. After 24 months of treatment, ≥92% of all treated groups had centrilobular or panlobular hepatocyte hypertrophy.. At both doses, 96% of males had single-cell hepatocellular necrosis ($p<0.05$) and significantly ($p<0.05$) increased mitotic figures and liver inflammation. Significantly ($p<0.05$) increased altered hepatocytes with basophilic foci were also seen in both sexes at the high dose.

C. CARC Conclusions:

From these data, CARC concluded that the mouse liver tumors, driven mainly by adenomas, were treatment-related in both sexes.

D. Mechanistic studies

A mode of action has been postulated by the Registrant for nitrapyrin-induced mouse liver tumors, which is mitogenesis through activation of the constitutive androstane receptor (CAR). The key events for the proposed MOA include the following: CAR activation followed by altered gene expression specific to CAR activation, increased cellular proliferation, altered hepatic foci, and development of tumors.

1. Previously reviewed studies

In the earlier assessment, CARC evaluated the mechanistic studies provided by the registrant to address the proposed mode of action (MOA) for mouse liver tumors. For clarity, only the male mouse data were considered because the mechanistic studies were generally done only with male mice. However, since no sex specific effect was identified, these deliberations apply equally to the females.

a) In the first mechanistic study (MRID 48210503), male B6C3F1 mice were administered nitrapyrin in the diet at 0, 75, 250 or 400 mg/kg/day for 7 or 14 days. Parameters evaluated included body weights, food consumption, clinical chemistry, liver weights, and histopathologic examinations. Additional studies with liver tissue investigated gene expression of targeted genes, cell proliferation (BrdU uptake), activity of the specific liver enzymes, [total P450 content and pentoxyresorufin O-dealkylase (PROD)], and Western immunoblotting.

Summarized results show that treatment groups given 250 or 400 mg/kg/day nitrapyrin for 7 or 14 days had significant increases in absolute and relative liver weights (**Table 3**) consistent with the histopathologic identification of centrilobular/midzonal hypertrophy (slight to moderate) with altered tinctorial properties of the cytoplasm (eosinophilia), and increased midzonal and periportal hepatocellular cell proliferation (**Table 4**). The analysis of hepatocellular proliferation via BrdU incorporation indicated a dose-(250 and 400 mg/kg), and duration-related induction of S-phase DNA synthesis (higher at 14 days vs. 7 days) (**Study Text Table 5, p.15**). Recovery group animals had no treatment-related liver weight increases (**Table 3**), histopathologic alterations (**Table 4**), or cell proliferation changes (**Study Text Table 5, p. 15**).

Table 3. Nitrapyrin (N-Serve™) $B_6C_3F_1$ Mouse Study: Absolute and Relative Liver Weights

Dose (mg/kg/day)	0	75	250	400
Males				
Terminal BW g±SD	24.5 ± 0.9	24.6 ± 0.8	24.3 ± 1.0	24.3 ± 1.1
Week 1				
Absolute (g)	1.348 ± 0.9	1.386 ± 0.8	1.593* ± 0.8 (+18%)	1.857* ± 0.2 (+38%)
Relative (%)	5.492 ± 0.2	5.634 ± 0.2	6.561 ± 0.2* (+19%)	7.637 ± 0.4* (+39%)
Week 2				
Absolute (g)	1.403 ± 0.6	1.419 ± 0.1	1.688 ± 0.1* (+20%)	1.973 ± 0.2* (+41%)
Relative (%)	5.456 ± 0.2	5.617 ± 0.2	6.761 ± 0.2* (+24%)	8.122 ± 0.4* (+49%)
Week 2 w/ 3 Week Recovery				
Absolute (g)	1.443 ± 0.1	1.420 ± 0.1	1.465 ± 0.1	1.513 ± 0.2
Relative (%)	5.341 ± 0.1	5.361 ± 0.1	5.503 ± 0.3	5.650 ± 0.3

% C = Percent from control
[a] Data obtained from pages 273-301 of MRID 49020024.
* Statistically different ($p < 0.05$) from the control.
** Statistically different ($p < 0.01$) from the control.

Table 4. Nitrapyrin (N-Serve™) $B_6C_3F_1$ Mouse Study: Selected Histopathological Findings

7-Day Treatment				
Dose (mg/kg/day)	**0**	**75**	**250**	**400**
Liver No. examined	6	6	6	6
Hypertrophy; hepatocyte; with altered tinctorial properties; centrioblular/midzonal				
Very Slight	0	0	2	0
Slight	0	0	4	6
Moderate	0	0	0	0
Mitotic alteration: increased hepatocyte; multifocal				
Very Slight	3	2	5	0
Slight	0	0	1	6

14-Day Treatment				
Dose (mg/kg/day)	**0**	**75**	**250**	**400**
Liver No. examined	6	6	6	9
Hypertrophy; hepatocyte; with altered tinctorial properties; centrioblular/midzonal				
Very Slight	0	0	1	0
Slight	0	0	5	0
Moderate	0	0	0	9

Mitotic alteration: increased hepatocyte; multifocal				
Very Slight	0	0	4	4
Slight	0	0	0	3

3-Week Recovery				
Dose (mg/kg/day)	**0**	**75**	**250**	**400**
Liver No. examined	6	6	6	9
Hypertrophy; hepatocyte; with altered tinctorial properties; centrioblular/midzonal				
Very Slight	0	0	0	0
Slight	0	0	0	0
Moderate	0	0	0	0
Mitotic alteration: increased hepatocyte; multifocal				
Very Slight	2	0	2	0
Slight	0	0	0	0

Data are derived from Study Report, p 31, MRID 48210503

Text Table 5. Summary of Hepatocellular Proliferation

BrdU Mouse Liver Proliferation: 7-Day Nitrapyrin Treatment				
	CL	**MZ**	**PP**	**Total**
Dose/Zone	Fold-Change	Fold-Change	Fold-Change	Fold-Change
Control	1.0	1.0	1.0	1.0
75 mkd	-1.5	-1.9	-1.8	-1.8
250 mkd	1.1	1.5	3.3*	2.0
400 mkd	1.9	3.0*	8.0*	4.3*
BrdU Mouse Liver Proliferation: 14-Day Nitrapyrin Treatment				
	CL	**MZ**	**PP**	**Total**
Dose/Zone	Fold-Change	Fold-Change	Fold-Change	Fold-Change
Control	1.0	1.0	1.0	1.0
75 mkd	1.0	-1.3	1.1	-1.1
250 mkd	2.1	1.7	3.8*	2.4*
400 mkd	1.2	3.8*	11.2*	4.9*
BrdU Mouse Liver Proliferation: 14-Day Nitrapyrin Treatment and 21-Day Recovery				
	CL	**MZ**	**PP**	**Total**
Dose/Zone	Fold-Change	Fold-Change	Fold-Change	Fold-Change
Control	1.0	1.0	1.0	1.0
75 mkd	1.1	1.0	-1.1	1.0
250 mkd	-1.0	1.2	1.2	1.1
400 mkd	-2.6	-3.0	-3.1*	-2.9
*Data are relative fold-change compared to control, * = statistically identified, Dunnett's test ($\alpha \leq 0.05$). Abbreviations: CL – centrilobular, MZ – midzonal, PP – periportal.*				

Data were extracted from the Study Report, Text Table 5, p. 33 MRID 48210503.

Gene expression analysis of the liver indicated a dose-related increase in the *Cyp*2b10/CAR-associated transcript levels (**Table 5, p.16**). Cyp2b10 expression was 351-fold higher than controls at the tumorigenic dose of 250 mg/kg/day after 7 days of treatment with nitrapyrin and 390X higher after 14 days. The increased transcript levels correlated with an increase protein levels as confirmed by the western blot analysis (**Text Figure 1, p.17**). These findings are consistent with direct activation of the CAR nuclear receptor. Recovery group animals had no treatment-related increases in *Cyp2b10*-induction. Gene expression analysis of AhR, PXR, or PPAR-α signaling pathways did not show convincing evidence that these three signaling pathways were activated in response to Nitrapyrin treatment. Although nitrapyrin induced Cyp2b10 transcript and protein levels, there was a lack of P450 enzyme induction (total P450) and *Cyp2b10*-mediated PROD activity (**Table 6**).

Table 5 - Summary of Targeted Gene Expression in Liver (Fold Increase) [a]

Gene Expression: 7-Day Nitrapyrin Treatment				
Dose/Gene	*Cyp1a1*	*Cyp2b10*	*Cyp3a11*	*Cyp4a10*
Control	1	1	1	1
75 mkd	1.11	4.05	0.86	1.32
250 mkd	1.96	351.02	1.38	6.75
400 mkd	2.03	716.04	1.51	5.19
150 mkd PB	2.06	807.43	6.93	1.13

Gene Expression: 14-Day Nitrapyrin Treatment				
Dose/Gene	*Cyp1a1*	*Cyp2b10*	*Cyp3a11*	*Cyp4a10*
Control	1	1	1	1
75 mkd	1.16	4.45	0.69	1.23
250 mkd	1.67	389.59	1.12	4.22
400 mkd	1.87	1092.32	1.19	2.91

Gene Expression: 14-Day Nitrapyrin Treatment and 21-Day Recovery				
Dose/Gene	*Cyp1a1*	*Cyp2b10*	*Cyp3a11*	*Cyp4a10*
Control	1	1	1	1
75 mkd	1.02	1.60	1.08	1.21
250 mkd	0.92	2.91	1.23	1.57
400 mkd	1.04	2.70	0.88	1.33

[a] All treatment group animals in the main study had 6 mice/group; all sample for the positive control (PB = Phenobarbital) had 5 samples/group). The positive control was not run concurrently with the main test. Data were derived from Table 14 of the Study Report (MRID 48210503).

Text Figure 1. Specific Cyp2b10 Western Blot for Representative Samples From Each Treatment Group

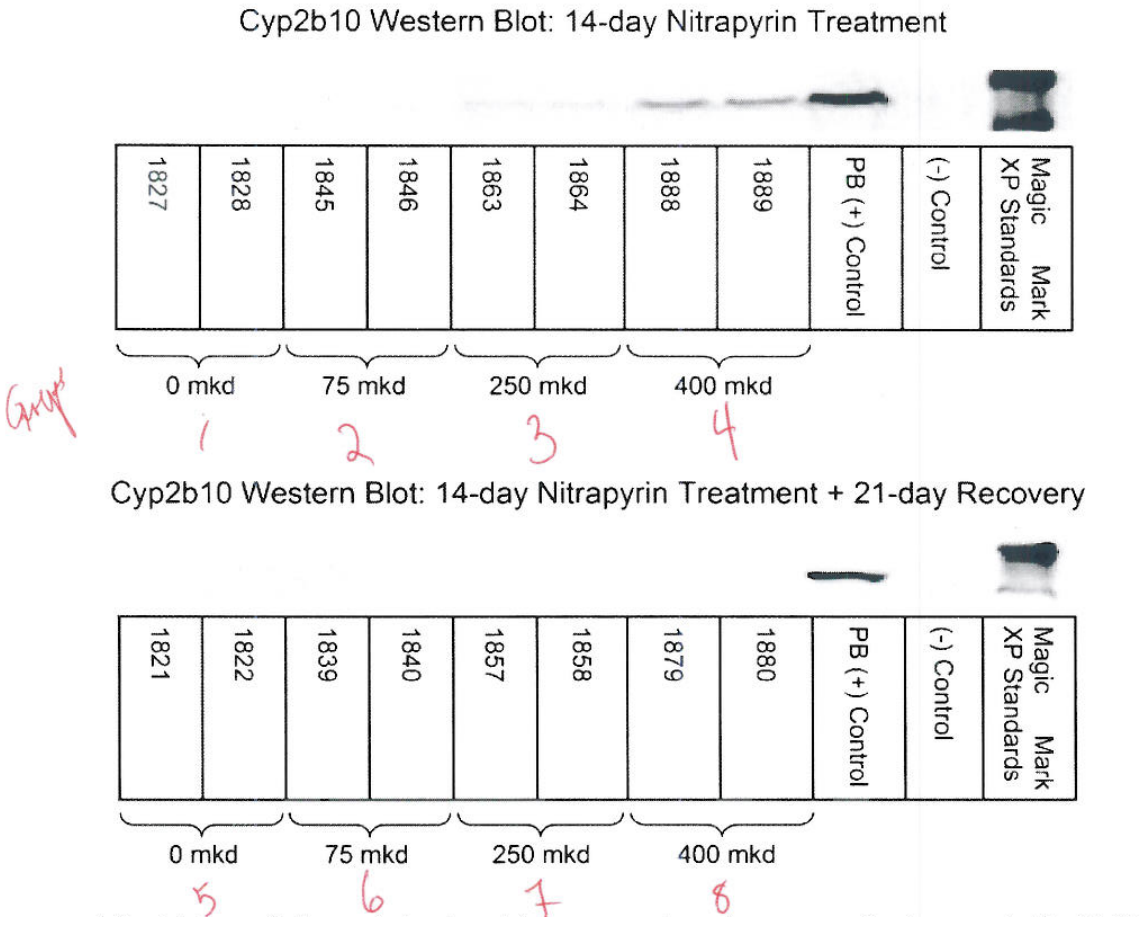


Table 6. Summary of Liver Enzyme Assays with Nitrapyrin (Pooled Samples)[a]

Group	Total Mean P450 Content (nmol p450/mg protein)	Fold Increase	PROD[b] (pmol/mL)	Fold Increase
14-day Exposure				
0 mg/kg/day	0.33±0.04	-	VNP[c]	1
75 mg/kg/day	0.31±0.03	<1	VNP	1
250 mg/kg/day	0.42±0.04	1.3	VNP	1
400 mg/kg/day	0.43±0.05	1.3	VNP	1.1
14-day Exposure/21-day Recovery				
0 mg/kg/day	0.32±0.05	-	VNP	1
75 mg/kg/day	0.32±0.03	1	VNP	1
250 mg/kg/day	0.47±0.05	1.5	VNP	1.1
400 mg/kg/day	0.49±0.04	1.5	VNP	1.1

[a] Data were derived from Appendix A, p. 193 and Table 8, p. 14, MRID 48210503.
[b] PROD = Pentoxyresorufin O-dealkylase
[c] VNP= Values not presented

b) Other studies in the nitrapyrin database were revisited for liver effects such as absolute and relative liver weights, histology, and retrospective analysis of cell proliferation and/or apoptosis (See Fourth CARC Report, dated March 1, 2012, TXR No. 0056262).

These data along with the mechanistic data from MRID 48210503 became central to the mouse liver tumor MOA analysis to identify and support the proposed key events; to assess the causal relationships and to judge the consistency of the data. From the overall analysis presented at the 4th Meeting, CARC concluded that the inadequate data on cell proliferation and the lack of PROD induction did not support the MOA for liver tumor induction proposed by the registrant. It was further stated that it may be prudent for the registrant to perform an additional study on cell proliferation at earlier times in the dosing regimen to better define the time course of the proliferative response. It was also suggested that a CAR null mouse assay be considered for better characterization of the MOA (Fourth CARC Report, 2012).

2. **New studies**

a) In response to the CARC recommendation, the registrant submitted an *in vivo* constitutive androstane receptor (CAR) knockout assay (MRID 49677501) conducted to investigate the mode of action (MOA) for liver tumors in male mice. Groups of six male C57BL/6NTac wild type (WT $CAR^{+/+}$)[1] and CAR knockout (CAR KO; $Car^{-/-}$) mice were fed diets containing 0 or 250 mg/kg/day[2] nitrapyrin (90.3%/Lot No. A6TET, TSN003679-0025) in the diet for 4 days. Treated mice were implanted with mini-osmotic pumps containing 5-bromo-2'-deoxyuridine (BrdU) 4 days prior to the scheduled sacrifice to ensure continuous infusion. Morbidity, mortality and clinical observations were performed twice daily. Body weights were recorded pre-testing and on test days (TDs) 1 and 5. Body weight gain was calculated after the weight of the osmotic pump was subtracted from the TD 1 and terminal body weights. Food consumption was determined on TDs 1 and 5. Necropsies were performed on TD 5; the liver and a 2-3 cm segment of the proximal duodenum were excised and prepared for histopathological examination. Hepatic S-phase DNA synthesis was determined using BrdU immunohistochemistry. Duodenal tissue from each animal served as individual positive controls for BrdU incorporation. Liver samples from each animal were processed for RNA extraction and gene expression responses for *Cyp1a 1, Cyp2b10, Cyp3a11,* and *Cyp4a10* were assessed as biomarkers for activation of AhR, CAR, PXR, and PPAR-α signaling pathways, respectively.

No deaths or clinical signs of toxicity were seen in either the WT or CAR KO mice fed diets containing 250 mg/kg/day for 4 days. Nitrapyrin had no adverse effects on body weight, body weight gain or food consumption. Absolute and relative liver weights for both mouse strains were significantly ($p \leq 0.05$) increased in nitrapyrin treated animals; differences for both strains were 17% for the absolute liver weights and 19% for the relative liver weights **(Table 7, p. 19)**. These liver weight changes were consistent with treatment-related histopathological alterations in the liver (*i.e.,* very slight increase in hepatocytes with mitotic figures, slight centrilobular/midzonal hepatocellular hypertrophy and very slight vacuolization of the centrilobular/midzonal hepatocytes) (**Table 8, p. 20**). The histopathological alterations were also in agreement with the previously reported liver findings in B6C3F1 mice treated with the tumorigenic dose of nitrapyrin (250 mg/kg/day) for 12 months in the repeat mouse carcinogenicity study (MRID 44231803).

Table 7. Absolute and Relative Liver Weights in Two C57L/6Tac Strains of Male Mice Fed Dietary Levels of Nitrapyrin[a]

Mouse Strain[b]	Doses (mg/kg/day)			
	0		250	
	Absolute Liver Weight (g)	Relative Liver Weight (g/100)	Absolute Liver Weight (g)	Relative Liver Weight (g/100)
Wild Type	1.357±0.103	5.810±0.230	1.632*±0.081(17%↑)	7.205*±0.306 (19%↑)

1 C57BL/6NTac wild type served as the surrogate mouse stain for the B6C3F1 mouse used in the earlier carcinogenicity studies (See Section I, Materials and Methods, A.3, Materials Test System).
2 Carcinogenic dose (MRID 44231803)

CAR Knock Out	**1.207±0.175**	**4.966±0.717**	**1.451*±0.075 (17%↑)**	**6.102*±0.275 (19%↑)**

[a] Data were extracted from study report Text Table 3, p. 27 Tables 7 and 8, pp. 43 and 44 (MRID 49677501).
[b] N = 6 males/dose
***** Significantly different from control ($p \geq 0.05$); Student t-test

Table 8. Histopathological Findings in Male WT or CAR KO Mice Fed Nitrapyrin in the Diet[a]

Mouse Type	**WT**		**CAR KO**	
Dose (mg/kg/day)	**0**	**250**	**0**	**250**
Liver: Number examined	6	6	6	6
Increased # of mitotic figures; hepatocyte				
Very Slight	1	5	0	0
Hypertrophy; increased eosinophilia; hepatocyte; centrilobular/mid-zonal				
Very Slight	0	0	0	6
Slight	0	6	0	0
Vacuolizatoin; consistent with fatty change; hepatocyte; centrilobular/mid-zonal				
Very Slight	0	5	0	6

[a] Data were extracted from the study report, Text Table 4, p.29 (MRID 49677501).

Gene expression data indicated a marked increase (494-fold) in the *Cyp2b10/*CAR-associated transcript levels in liver tissue following nitrapyrin treatment in WT animals compared to a minimal increase observed in CAR KO mice treated with nitrapyrin (**Table 9, p. 20**). These findings confirm the results from an earlier mechanistic study (MRID 48210503) showing a 350-fold or a 390-fold increase in *Cyp2b10* expression in B6C3F1male mice after 7 or 14-days of treatment with 250 mg/kg/day nitrapyrin, respectively, as well as the western blot analysis of the CYP2b protein in the liver tissue of B6C3F1male mice (**Figure 1, p.17**).

Table 9. Summarized Results of Gene Expression Analysis Performed on the Livers of WT and CAR KO Male Mice fed a Diet Containing Nitrapyrin[a]

Dose/Gene	**Cyp1a1**	**Cyp2b10**	**Cyp3a11**	**Cyp4a10**
Control	1	1	1	1
250 mg/kg/day (WT)	2.7	493.7	22	1.5
250 mg/kg/day (CAR KO)	104.4	2.2	2.6	2.6

[a] Data were extracted from study report Table 5, p.30 (MRID 49677501).

WT mouse livers showed a significant ($p \leq 0.05$) increase in the BrdU labeling index (LI) following nitrapyrin treatment. In the periportal region of the liver, the increase in BrdU LI in the treated WT mice (**Table 10, p. 21**) was 2.4 vs. <1 in treated CAR KO mice (**Table 11, p. 21**). These data also confirm the earlier results of significant ($p<0.05$) 3.3- to 3.8-fold increases in BrdU LIs in the periportal region of the liver from the 7- or 14-day mechanistic study **(Text Table 5, p. 15)** mentioned above with B6C3F1male (MRID 48210503). By contrast, no increases in BrdU LIs were observed in the CAR KO male mice at 250 mg/kg/day.

Table 10. Summary of Hepatocellular Proliferation (BrdU Labeling Indices) of Lobular Zones in the Livers of WT Male Mice Treated with Nitrapyrin in the Diet[a]

Dose/Gene	CL	MZ	PP	Total
Nitrapyrin				
0	1.0	1.0	1.0	1.0
250	0.8	1.4	2.4*	1.5*

[a] Data were extracted from study report Text Table 6, p. 31 (MRID 49677501).

Table 11. Summary of Hepatocellular Proliferation (BrdU Labeling Indices) by Lobular Zones in the Livers of CAR KO Male Mice Treated with Nitrapyrin in the Diet[a]

Dose/Gene	CL	MZ	PP	Total
Nitrapyrin				
0	1.0	1.0	1.0	1.0
250	0.6	0.8	0.9	0.8

[a] Data were extracted from study report Text Table 7, p. 31 (MRID 49677501).

Overall, CARC concludes that the data from the currently reviewed study provide reproducible evidence of direct activation of the CAR nuclear receptor as evidenced by the marked induction of *Cyp2b10* gene expression by nitrapyrin in the male C57L/6Tac Wild Type mice, surrogate strain for B6C3F1 but not in the male CAR KO strain.

b) In an *in vitro* cell proliferation assay (MRID 49677502), replicate cultures of mouse or human primary hepatocytes (derived from the livers of two mice or two human subjects) were exposed for 48 hours to 0, 1, 3, 10, 30, or 100 µM[3] Nitrapyrin (90.3%/Lot No. A6TET, TSN003679-0025), prepared in dimethyl sulfoxide (DMSO). The outcome of this study is discussed in detail in Section V (Human Relevance of Nitrapyrin Mouse Liver Tumors).

c) In response to the CARC's comment that PROD induction did not support the MOA for liver tumor, the registrant also submitted an *in vitro* mechanism-based (suicide) inhibition assay (MRID 49677503). In this study, commercially prepared phenobarbital (PB)-induced rat liver microsome along with the fluorometric substrate (7-pentoxyresorufin-O-dealkylase (PROD) was incubated with 0, 1, 10, 50, 100 or 500 µM[4]of nitrapyrin (90.3%/Lot No. A6TET, TSN003679-0025), prepared in dimethyl sulfoxide (DMSO) for 20 minutes in duplicate microtitre plates. PROD activity was measured using a fluorometric plate reader. The positive control for this test system was 10 to 80 µM curcumin and the negative control was 1 to 500 µM PB.

Nitrapyrin inhibited PROD activity in a concentration-related manner reducing activity from 45-59% at 10 µM to 4-5% at 500 µM **(Table 12, Figure 2**) in the two runs reported. By contrast, PB had no effect on PROD activity and the positive control, curcumin, induced a concentration-dependent inhibition of PROD activity.

It was, therefore, concluded that nitrapyrin induced inhibition of PROD enzyme activity in rat microsomes. However, this does not answer the question of PROD activity in mouse microsomes. Nevertheless, these data are extraneous to the MOA analysis because CAR-

[3]Equivalent to 0, 0.25, 0.76, 2.5, 7.6, or 25 ng/mL, respectively
[4]Equivalent to 0, 0.25, 2.5, 12.5 25, or 125 ng/mL, respectively

mediated activity is confirmed by the gene expression data presented in Tables 5 (B6C3F1 mice) and 8 (CAR WT mice) and Figure 1.

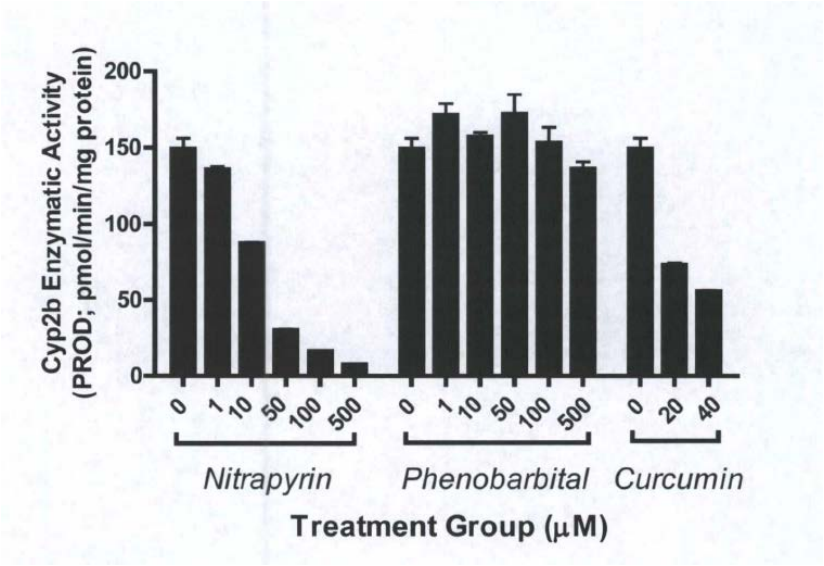
Table 12. In Vitro Enzymatic Activity (PROD) of PB-Induced Rat Liver Microsomes[a]

	Run 1		Run 2		Run 3	
	Activity ± SD (pmol/min/mg protein)	Activity (% of control)	Activity ± SD (pmol/min/mg protein)	Activity (% of control)	Activity ± SD (pmol/min/mg protein)	Activity (% of control)
Solvent control (0.1% DMSO)	175.1 ± 5.0	100	196.2 ± 1.2	100	149.0 ± 6.9	100
1 µM nitrapyrin			168.8 ± 6.9	86.0	135.8 ± 1.7	91.2
10 µM nitrapyrin			87.3 ± 3.8	44.5	87.4 ± 0.6	58.7
50 µM nitrapyrin			24.8 ± 0.1	12.6	30.0 ± 0.7	20.2
100 µM nitrapyrin			16.7 ± 0.2	8.5	16.1 ± 0.2	10.8
500 µM nitrapyrin			7.9 ± 0.0	4.0	7.5 ± 0.1	5.1
10 µM curcumin	134.8 ± 3.1	77.0	156.5 ± 0.5	79.8		
20 µM curcumin	107.4 ± 3.7	61.3	132.1 ± 0.8	67.3	72.7 ± 1.5	48.7
40 µM curcumin	86.8 ± 5.9	49.6	96.5 ± 3.3	49.2	55.5 ± 0.7	37.2
80 µM curcumin	41.7 ± 3.2	23.8	89.3 ± 5.5	45.5		
1 µM phenobarbital					171.0 ± 7.5	114.8
10 µM phenobarbital					156.7 ± 3.1	105.2
50 µM phenobarbital					171.7 ± 12.9	115.3
100 µM phenobarbital					152.9 ± 10.0	102.6
500 µM phenobarbital					136.1 ± 4.7	91.3

+

DMSO= Dimethylsulfoxide, SD= standard deviation; PROD = 7-Pentoxyresorufin-O-dealkylase
[a]Table taken from Table 1, page 13 of study report (MRID 49677503).

Figure 2. Inhibition of CYP2B-Mediated Metabolic Activity of PB-Induced Rat Liver Microsomes[a].



[a]Figure taken from Figure 1, page 12 of study report (MRID 49677503).

IV. APPLICATION OF THE CANCER GUIDELINES MODE OF ACTION (MOA) FRAMEWORK

A. Postulated MOA and Key Events: Portions of the following narrative were extracted from the fourth CARC review (TXR No. XXXX). Additionally, the registrant submitted an MOA and human relevance framework document for mouse liver tumors (MRID 49677504).

Based on the available information, the registrant's representatives postulated that the MOA for nitrapyrin-induced mouse liver tumors is "comparable to the MOA for phenobarbital-induced rodent liver tumors" (MRID 49677504). Accordingly, the MOA proposed by the registrant is **mitogenesis through activation of the constitutive androstane receptor (CAR)**.

Key events for this MOA include the following:

CAR Activation
Increased *Cyp*2b10 expression
Increased Hypertrophy
(Associative Event)[5]
Increased Liver Weight
(Associative Event)

[5] Biological process that is, of itself, not a causal, key event for an MOA but is a reliable indicator or marker for key events.

Increased Cell Proliferation
Liver Tumors

B. Support of Key Events/Causal Relationship between Key Events and the Tumor Response/ Consistency of the Data

Data supporting the key events from the earlier analysis in the fourth CARC report (TXR No. XX) along with the new data from the CAR knockout study have been incorporated into **Table 13 (pp. 26-27).** These data summarize key and associative event from feeding studies of varying duration (4 days up to chronic exposure), along with an analysis of the causal relationships relative to dose and time. The coherence of these data are also discussed in this section of the document.

The molecular initiating event [*i.e.*, the early point of chemical-biological interaction with the organism that initiates the adverse outcome pathway (AOP)], which is proposed by the registrant, is CAR activation. As shown in Table 13, marked and dose-related overexpression of the *Cyp*2b10 gene, the gene specific for CAR activation (Dickins, 2004), at the tumorigenic dose (250 mg/kg/day) was seen, showing a 351-fold and 390-fold increase in expression at weeks 1 and 2, respectively.

When the new data from the CAR knockout mouse assay (MRID 49677501) are added to Table 13, clear and marked activation of the *Cyp2b10* (CAR-associated) gene expression (494 X control) was seen in the WT after 4 days of treatment. By contrast, CAR KO male mice showed minimal or no induction of *Cyp2b10.* Increased liver hypertrophy and liver weights were also seen in a number of studies with B6C3F1 mice noted and also in both the WT and the CAR KO mice. The response for both events was initiated at week 1 or day 4, respectively, at 250 mg/kg/day. Since these responses were elicited in the $CAR^{-/-}$ mice as well as the $CAR^{+/+}$ and B6C3F1 mice, both events are considered associative rather than key events.

The key event, hepatocellular cell proliferation was increased in the B6C3F1 mice (2-fold by 1 week) to a magnitude comparable to that observed at the tumorigenic dose in WT mice (2.4-fold at 4 days). By contrast, no evidence of cell proliferation was observed in the CAR KO mice.

Liver tumors appeared 96 weeks post-treatment with 250 mg/kg/day nitrapyrin; long after all key and associative events were manifested at the tumorigenic dose. Although tumors were also induced at 125 mg/kg/day, CAR activation was not studied at this level. However, liver hypertrophy, increased liver weights and increased liver cell proliferation were apparent at 48 weeks. While it appeared from **Table 13 (p. 25, 26)** that there was a 4-fold increase in CYP2b10 expression at 75 mg/kg/day, no expression of the CYP2b10 protein was seen in the CYP2b10 Western Blot analysis. This evidence along with the lack of liver hypertrophy and cell proliferation are consistent with the absence of tumors at this level.

Table 13. Summarized Key Events Associated with Tumor Induction in Male Mice Fed Diets Containing Nitrapyrin									
Key Event	**Dose (mg/kg/day)**	**Days**	**Weeks**						**Reference MRID No.**
		4	**1**	**2**	**3[a]**	**12**	**48**	**96**	
	CAR[b] Activation (Key Event)								
↑*Cyp*2b10	75		4X[c]	4.5X[c]	2X[c]				48210503
	250		**351X**	**390X**	**3X**				
	400		716X	1092 X	3X				
CAR$^{+/+}$ Knockout Wild Type	**250**	**494X**							49677501
CAR$^{-/-}$ Knockout	**250**	**2.2x**							
	↑Liver Hypertrophy (Associative Event)								
	75		0%[d]	0% [d]	0% [d]				48210503
	125						100%	50%	44231803
	200					100% [d]			44231802
	250		**83%**	**100%**	**0%**				48210503
	250					**80%**	**49%**		44231803
	300					100%			44231802
	400		83%	100%	0%				48210503
	400					100%			44231802
CAR$^{+/+}$ Knockout Wild Type	**250**	**100% [d] (slight)**							49677501
CAR$^{-/-}$ Knockout	**250**	**100% (very slight)**							49677501

Table 13. Summarized Key Events Associated with Tumor Induction in Male Mice Fed Diets Containing Nitrapyrin									
Key Event	**Dose (mg/kg/day)**	**Days**	**Weeks**						**Reference MRID No.**
		4	**1**	**2**	**3[a]**	**12**	**48**	**96**	
	↑Liver Weight (Associative Event)								
	125						(24%)	(24%)	44231803
	200					(25%)			44231802
	250		**(19%)**	**(24%)**	**(1%)**				48210503
	250						(24%)	(101%)	44231803
	300					(49%)			44231802
	400		(39%)	(49%)	(1%)				48210503
	400					(70%)			44231802
$CAR^{+/+}$ Knockout Wild Type	**250**	**17%A /19%R**							49677501
$CAR^{-/-}$ Knockout	**250**	**17%A /19%R**							
	↑Cell proliferation (Key Event)								
	75		1X [c]	1X [c]	1X [c]				48210503
	125						2.5X [c]		44231803
	200					2-3X [c]			44231802
	200		17X						44231803/ 48231801
	250						**12X**		44231803
	250		**2X**	**2X**	**1X**				44231803
	400		4X	5X	<1X				
	400					8X			48210506
	400			105X					44231803/ 44231801

Table 13. Summarized Key Events Associated with Tumor Induction in Male Mice Fed Diets Containing Nitrapyrin									
Key Event	**Dose (mg/kg/day)**	**Days**	**Weeks**						**Reference MRID No.**
		4	**1**	**2**	**3[a]**	**12**	**48**	**96**	
CAR Knockout Wild Type	**250**	**2X**							49677501
CAR$^{-/-}$ Knockout	**250**	<1X							
Tumors (Combined) [f]	125							40%	44231803
	250							**94%**	

[a] Animals were dosed with the test material for 14 days and allowed a 21-day recovery period.

[b] Abbreviations: CAR = Constitutive androstane receptor

[c] Fold increase [d] % ↑ compared to control

[e] A= absolute/ R= relative

[f] Combined adenomas and carcinomas; lower doses (5, 20 or 75 mg/kg/day) showed no increases in tumors (MRID 4165160).

Bolded values = Response at the tumorigenic dose. New data from MRID 49677501 are presented in the grey rows.

C. Biological Plausibility and Coherence of the Data

The inclusion of the new data from the male CAR knockout assay (MRID 49677501) provides clear, dose-related evidence of *Cyp2b10* induction and cell proliferation, associated with 100% of the WT mice with increased liver hypertrophy and increased liver weights at the tumorigenic dose within 4 days of treatment. By contrast, little or no activity was seen in the CAR KO mice. These findings, in conjunction with evidence from a stop/recovery experiment (MRID 48210503; see fourth CARC report; Kidwell, 2011; TXR No. 0056262) that indicate that animals, treated for 2 weeks with 250 or 400 mg/kg/day and allowed a 21-day recovery, showed no adverse effects on cell proliferation, liver weights or histopathologic alteration, are supportive of a CAR-mediated MOA for nitrapyrin. Similarly, the MOA described for nitrapyrin is consistent with the well-known MOA for PB in rodents (Cohen, 2010; and Elcombe *et al.*, 2014). However, no MOA data were available for the female mice. Overall, the dose-related key events that have been observed in multiple mouse studies in the nitrapyrin database, in conjunction with the absence of the key events in the CAR KO male mice add considerable weight to the plausibility and coherence argument. It was concluded, therefore, that the weight of the evidence supports the CAR activation MOA for nitrapyrin for males mice only. No conclusions are possible for the female mice which has tumors at a lower dose than the males.

D. Alternative MOAs

Alternative MOAs (*i.e.*, DNA reactivity, cytotoxicity with regenerative cell proliferation, and mitogenesis induced by other nuclear receptors such as AhR, PXR, or PPAR α), which were evaluated in the fourth CARC report (Kidwell, 2011; TXR No. 0056262), have not changed and are still ruled out as possible alternative MOAs.

E. Uncertainties

Uncertainty regarding the absence of hepatic metabolic enzyme activity (PROD) in treated mice has not been fully resolved by the data presented from the suicide inhibition of PROD assay in rat microsome (MRID 49677503). Since PROD activity is an additional marker of CAR activation, however, CAR-mediated activity is confirmed by the gene expression data presented in Tables 5 (B6C3F1 mice) and 8 (CAR WT mice) and **Figure 1**. Furthermore, the uncertainty regarding the inadequate data on cell proliferation, which was identified in the previous CARC meeting as a deficiency in the MOA data, has also been resolved by the newly submitted data from the CAR knockout assay (MRID 49677501). In this study, we see a comparable (≈2-fold) increase in the proliferative response of the B6C3F1 and the CAR WT mouse livers to 250 mg/kg/day nitrapyrin juxtaposed by the absence of a response in the CAR KO mice. Nevertheless, uncertainty exists for the female mice with significntly tumor induction at a lower dose than the males.

V. COMMITTEE'S ASSESSMENT OF THE WEIGHT-OF-THE-EVIDENCE FOR THE CAR MOA

On May 25, 2016, CARC reconvened to evaluate the submissions of new studies containing MOA data on nitrapyrin; the new studies included: 1) Hepatic responses in male C57BL/6NTac wild type and CAR knockout mice (MRID 49677501); 2) *In vitro* analysis of mouse and human primary hepatocyte cell proliferation assay (MRID 49677502); and 3) *In vitro* investigation of suicide inhibition of hepatic CYP2B enzyme activity (MRID 49677503). From these deliberations, the CARC drew the following conclusions for the nitrapyrin-induced mouse liver tumor MOA:

- **There is reproducible evidence of direct activation of the CAR nuclear receptor as evidenced by the marked induction of *Cyp2b10* gene expression by nitrapyrin in the B6C3F1 male mouse strain, the C57L/6Tac Wild Type male strain, (surrogate strain for B6C3F1) but not in the CAR KO male strain.**

- **There is good concordance between the dose causing tumors (250 mg/kg/day nitrapyrin) and the dose response and temporal associations for the key and associative events.**

- **The dose-related key events that have been observed in multiple mouse studies in the nitrapyrin database as well as in CAR WT mice, in conjunction with the absence of the key events in the CAR KO mice add considerable weight to the plausibility argument.**

- **Alternative MOAs (*i.e.,* DNA reactivity, cytotoxicity with regenerative cell proliferation, and mitogenesis induced by other nuclear receptors such as AhR, PXR, or PPAR *α*) have been ruled out.**

- **There is plausible evidence that mode of action (MOA) for the mouse liver tumors induced by nitrapyrin is mitogenesis through activation of the constitutive androstane receptor (CAR) nuclear receptor but this conclusion only applies to the male mice.**

VI. CLASSIFICATION OF CARCINOGENIC POTENTIAL

In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, *2*005), the CARC concluded that **Nitrapyrin should remain classified as "Suggestive Evidence of Carcinogenic Potential".** The rationale for this decision is as follows:

- The liver tumor response induced by nitrapyrin occurred in male and female mice; no liver tumors were seen in the guideline 2-year carcinogenicity study in rats.

- The tumor response in both sexes occurred late in the course of treatment and was driven by adenomas.

- Mutagenicity has been ruled out as an MOA for this response.

- Other alternative MOAs (cytotoxicity and regenerative cell proliferation, mitogenesis induced by other nuclear receptors such as PXR, AhR, or PPAR-α) have been ruled out.

- There is reproducible evidence of direct activation of the CAR nuclear receptor as evidenced by the marked induction of *Cyp2b10* gene expression by nitrapyrin in the B6C3F1 mouse strain and the C57L/6Tac Strains Wild Type (surrogate strain for B6C3F1) but not in the CAR KO strain.

- Similarly, the new data show time and dose concordance for all of the key events at the tumorigenic dose of 250 mg/kg/day nitrapyrin.

- The weight of the evidence suggests that the MOA for male mouse liver tumors is mitogenesis through activation of the constitutive androstane receptor (CAR) nuclear receptor. No conclusions regarding the MOA for liver tumors in female mice can be reached, with tumor induction evident at a lower dose than the male mice.

VII. QUANTIFICATION OF CARCINOGENIC POTENTIAL

The CARC determined that quantification of cancer risk using a non-linear approach (i.e., RfD) would adequately account for all chronic toxicity, including carcinogenicity, that could result from exposure to nitrapyrin.

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